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(54) Biospecific assay method.

(57)   This invention relates to a biospecific assay method, in which microparticles coated with the bioaffinity reactant A binding the analyte to be assayed ; the sample to be analyzed, and the labelled bioaffinity reactant B are mixed. After the binding reaction the signal strength from the labelled bioaffinity reactant B bound to the microparticles is quantitated for the determination of the concentration of the analyte in the sample. According to the invention, such an amount of sample and microparticles is used in the assay that after binding of the analyte of the sample to the said amount of microparticles, each individual microparticle will emit such a signal strength as to allow the measurement of the analyte concentration over the whole range of typical analyte concentrations, and the signal strength from each microparticle is measured separately.

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This inv ntion relates to a biospecific assay m thod in which the analyte to be assay d is bound t the surface of microparticles through a bioaffinity reactant binding the aforesaid analyt , and th am unt f analyte bound to individual microparticl s is d t rmined.

5   BACKGROUND OF THE INVENTION

The publications and other materials used herein to illuminate the background of the invention, and in particular, cases to provide additional details respecting the practice, are incorporated by reference.

Traditionally, radioimmunoassays were in the beginning performed in solution using test-tubes and com-
10   plicated separation methods. With the advent of solid phase technology radioactive isotopes have been replaced by diverse labels, and especially monoclonal antibodies have been used in an increasing number of applications.

The aforementioned improvements have made a wider range of traditional immunoassays and other bioaffinity assay methods available (e.g. Alternative Immunoassays, W.P. Collins, Wiley, Chichester 1985, and
15   Luminescence Immunoassay and Molecular Applications, Ed. Knox van Dyke, CRC Press, Boston 1990, and R.P. Ekins et al., Clin. Chem. 1991; 37:1955 - 1967). Solid phase assays have become routine assay methods both in the field of competitive and non-competitive assays, and the traditional test-tube has often been replaced by alternative solid phase facility. The microtiter plate was introduced as a solid phase for routine hormone assays in 1984 when Wallac Oy (Turku, Finland) introduced their DELFIA technology. Other commercial
20   enterprises have also adopted the microtiter plate in similar routine assays, which, however, are based on different labelling technologies.

Microparticles manufactured of different materials have for some time been available as an alternative solid phase for bioaffinity assays (e.g. Molday W.J. et al., J. Cell. Biol.: 1975: 64, 75, and Nustad K. et al.: Microspheres. Medical and biological applications, Ed. A. Rembaum and Z.A. Tökes, Boca Raton, Florida, CRC
25   Press 1988). Compared to microtiter plates the microparticles offer several advantages, for instance an immobilization method for bioaffinity reactants more easily adaptable to production scale, and a greater reaction velocity obtainable when using microparticles. The capacity of the microsphere-associated solid phase is also easily controllable by simply dispensing an amount of particles optimal for the method in question. The greatest disadvantages of microparticles have been traditionally associated with liquid handling (e.g. washes). With the
30   introduction of magnetic microspheres this problem has been partly overcome, and in recent years an increasing number of commercial automated immunoassay methods have been based on their use. Nowadays novel manufacturing methods have allowed the production of microparticles of exact and reproducible size (e.g. of a diameter of 0.04 - 100 μm). Also the immobilization of the bioaffinity reactants onto the microparticles can be effected by various chemical methods.
35   The competitive biospecific assay method may be described as follows: The microparticles are coated with the bioaffinity reagent A, for whose binding sites, on the one hand, the analyte contained in the sample competes, and on the other hand, the bioaffinity reagent B, in this case an appropriately labelled analyte, also competes for the said binding sites. In the case of immunoassay, bioaffinity reagent B will be a labelled antigen, the antigen being the same as the antigen contained in the sample or to a suitable labelled derivative thereof.
40   Because the analyte contained in the sample and the labelled analyte compete for the binding sites of the bioaffinity reactant A, the amount of label bound to the microparticle via reactant A, and consequently the signal from the microparticle, will be inversely related to the concentration of the analyte in the sample.

The non-competitive biospecific assay method may be described as follows: The microparticles are coated with the bioaffinity reactant A, whose binding sites only bind the analyte contained in the sample. The bioaffinity
45   reactant B, which in this case is a suitably labelled reactant directed against the analyte contained in the sample, is bound by the analyte, which is linked to the microparticle via the bioaffinity reactant A. In the case of immunoassay, bioaffinity reactant B will be a labelled antibody directed against the antigen contained in the sample. The amount of label bound to the microparticle via reactant A, and consequently the signal strength from the microparticle, will be directly related to the concentration of the analyte in the sample.
50   The sensitivity of the competitive and non-competitive immunoassay has been extensively discussed earlier (Ekins R.P. et al., Pure and Appl. Chem., 1985; 57: 473 - 482, and Ekins R.P. et al., Clin. Chem., 1991; 37: 1955 - 1967). As regards the present invention, it is essential to consider the factors affecting the sensitivity and functionality of each assay principle.

Maximal s nsitivity in a competitiv immun assay when using an antibody (a bioaffinity reactant) with an
55   affinity constant K will be obtained by dividing by K th relative rror of the signal strength at zero concentration. Th error of the signal strength will be affected by two components, an experimental rror compon nt du to pipetting and th r manipulations, and a signal str ngth measurement component, .g. th statistical rror of signal strength measurement. For th sak of simplicity, let us assume that th rror due to signal

strength measurement is 0, which is usually th case wh n the specific activity of th label is high. The maximal attainable sensitivity f th competitiv assay is n w ∈/K wh re ∈ is the combined relativ rror du to xper- imental factors. Let us assum , for instance, that the xperimental rror is of th order of 1%; th maximal attainable sensitivity then, using an antibody with an affinity constant as high as possibl , f r instance,

5 10^{12} l/mol, is of the order of 0.01 picomol/l. The sensitivity definition also demonstrates that unless the exper- imental error can be almost completely eliminated (< 0.1%), there is no sensitivity advantage to be gained in using a label with extremely high specific activity.

Likewise, the factors affecting the sensitivity of the non-competitive are the following:

a) the relative error of the signal strength (α) when the analyte is omitted, i.e., the error due to non-specific
10 binding of the labelled antibody (bioaffinity reactant)

b) the relative amount of non-specifically bound labelled antibody (k), and

c) the affinity constant (K) of the antibody The sensitivity of the non-competitive assay is then k/K x α. The sensitivity of both competitive and non-competitive assay is inversely related to the affinity constant of the antibody used, but the non-competitive assay is affected by the relative error due to, on the one
15 hand, non-specific binding of the labelled antibody and on the other hand, the relative binding of the lab- elled antibody, both of which could be assigned, for instance, a value of 1%. The maximal sensitivity of the non-competitive assay is then 0.0001 x 1/K or 0.1 fmol/l, if the affinity constant of the antibody used is 10^{12} l/mol, which is usually considered to be the highest possible value. In a non-competitive assay it is advisable to consider the specific activity of the label, because it affects factors a) and b), and offers a
20 possibility to improve the sensitivity of the assay.

In assays based on bioaffinity reactions factors structurally limiting the sensitivity of various determina- tions must also be considered; this is also essential as regards the present invention, because measurements will be taken from individual microparticles with extremely small amounts of analytes present.

25 ## SUMMARY OF THE INVENTION

This invention relates to a biospecific assay method, in which method microparticles coated with the bio- affinity reactant A binding the analyte to be assayed; the sample to be analysed, and the labelled bioaffinity reactant B are mixed. After the binding reaction the signal strength from the labelled bioaffinity reactant B
30 bound to the microparticles is quantitated for the determination of the analyte concentration of the sample. According to the present invention such an amount of sample and microparticles is used in the assay that after binding of the analyte of the sample to the said amount of microparticles, each individual microparticle will emit such a signal strength as to allow the measurement of the analyte concentration in the sample over the whole range of typical analyte concentrations, and the signal strength from each microparticle will be measured sep-
35 arately.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows a calibration curve for hCG construed by measuring individual microparticles by time-
40 resolved microfluorometry

Figure 2 shows the signal strength versus analyte concentrations for different amounts of microparticles (measured from individual microparticles)

Figure 3 shows the signal strength versus amount of microparticles per assay for two different amounts of analyte DNA present in the reaction (measured from individual microparticles). The curve indicated by
45 closed squares represents 10^9 molecules per reaction and the curve indicated by open squares represents 10^{10} molecules per reaction.

DETAILED DESCRIPTION OF THE INVENTION

50 The present invention allows the assay of the analyte in the sample at a minimal analyte concentration and in a minimal volume by selecting such an amount of microparticles that allows the measurement of the analyte bound to the surface of an individual microparticle, by the sensitive label technology used. The bio- specific reaction may be an immunoassay, a nucleic acid hybridization assay, a ligand-lectin assay or a ligand- receptor assay. The bioaffinity reacti n may b advantage usly performed on a micropartid having a diam-
55 eter of less than 1 mm. Th analyte concentrati n on the surface of the microparticle is d termined by means of th labelled bioaffinity reactant B, th label being n mitting fluorescence, time-resolved fluorescence, ch miluminescence r bi luminescence. The present inv nti n allows the measurement of both th highest and the l west concentrati ns of analyte from individual microparticles. Th sensitivity of th assay and th

measurement range ar controlled by th am unt of microparticles used in the assay.

Th pres nt inv ntion may be applied to many types of biospecific affinity reactions, although the most commonly used is the immunoch mical r acti n. This is why the present inv ntion is pred minantly described as a immunochemical assay. The relevant biospecific affinity reactions (immun reactions) are ither compet-
5 itive or non-competitive. Both immunoassay principles are commonly known. The factors affecting the sensi-tivity of the said immunoassay methods are known and the optimal sensitivity of the assays can be calculated. The highest attainable sensitivity in the competitive assay is about 10 fmol/l. The sensitivity cannot be im-proved by increasing the specific activity of the label used. Sensitivity can be improved to a certain degree by using a minimal antibody concentration. In the competitive assay the signal strength from the label to be
10 measured is inversely related to the concentration of the sample (analyte) to be assayed.

The highest possible sensitivity attainable in the non-competitive assay is about 0.1 - 0.01 fmol/l. The sen-sitivity may be enhanced if a label with a very high specific activity is available. The sensitivity can be improved up to a certain limit by using the highest possible concentration of labelled antibody if the background of the assay (non-specific binding to the surface) remains low. In the non-competitive assay the signal strength from
15 the label to be measured is directly related to the concentration of the sample (analyte) to be assayed.

In the competitive immunoassay the assumption may be made that the measuring range of the analyte to be assayed is typically 100-fold, i.e., so wide that the ratio of the highest to the lowest measured value is 100. In the competitive assay the lowest concentration gives the strongest signal and the highest concentration gives the weakest signal. In the competitive assay based on the use of microparticles, in which the measure-
20 ment is taken from individual particles, one must be able to measure the signal strengths from both the lowest and the highest concentration. If we assume a measuring range of 1 - 0.01 pmol/l for the sample to be assayed, then a concentration of 0.01 pmol/l corresponds to the strongest signal, and 1 pmol/l to the the weakest signal, respectively. The èoncentration of the analyte of the example is 1 - 0.01 attomol/µl which means that when using a sample volume of 1 µl (1 - 0.01 attomol of analyte to be assayed) one must be able to measure, from
25 a single microparticle, both the highest (0.01 attomol of sample) and the lowest (1 attomol of sample) signal strength. This means that when the concentration of the competing analyte is low, the highest possible number of analyte, i.e. bioaffinity reactant molecules will specifically bind to the surface of a single microparticle coated with a specific antibody, i.e. a second bioaffinity reactant. The lowest analyte concentration means that a sam-ple volume of 1 µl contains approximately 6,000 molecules of the analyte to be assayed, while the highest con-
30 centration corresponds to approximately 600,000 molecules in a microlitre, respectively. It is typical of the com-petitive assay that the signal strength decreases from about 100% to about 5 - 25% with increasing analyte concentration. The amount of the microparticles and of the analyte-specific antibody coated onto them will be adjusted to a minimal sample volume in order to allow a replacement (of labelled analyte by the analyte to be assayed) of 75 - 95% at the highest sample concentration. This still allows the remaining signal strength to
35 be reproducibly measured, by the label technology used, from the surface of individual microparticles, and at the same time, the signal strength (due to the specific binding of labelled analyte to the coated microparticle) corresponding to the lowest analyte concentration will not exceed the binding capacity of individual particles. The measuring range is adjusted for different assays by altering the amounts of the microparticles and anti-bodies used in the assay method in such a manner that measurements can always be taken from individual
40 microparticles.

The sensitivity of the non-competitive immunoassay is, at best, 0.01 - 0.1 fmol/l, and the measuring range is typically more than 100-fold, i.e. the ratio of the highest to the lowest measurement value is greater than 100. The signal strength measured in the assay is directly related to the concentration of the analyte to be assayed. For a required sensitivity of 0.01 pmol/l for the analyte to be assayed, and assuming a measuring
45 range of 1,000-fold, a sample volume of 1 µl will contain 0.01 - 10 attomol of the analyte to be assayed. If the sensitivity of the label technology is about 6,000 molecules per one microparticle, and with a signal strength directly related to the concentration of the analyte, it is possible for one microparticle to bind the analyte con-tained in 1 µl, and the amount of the analyte can be measured. The amount of microparticles used in the assay can be increased without changing the sample volume, if the sensitivity of the label techonology is better or
50 if a greater number of labelled molecules are linked to the labelled analyte-specific antibody (bioaffinity reac-tant). Accordingly, if the sensitivity of the label technology is lower, the sensitivity of the assay will decrease or the amount of analyte required on the surface of a single microparticle must come from a larger sample volume, which however limits the measurement range. The amount of the microparticles used in the assay, coated with the analyte-specific antibody or bioaffinity reactant as w ll as th amount of th analyte per mi-
55 croparticle will be adjusted s that a minimal concentration and volum f the analyte will contain en ugh ana-lyte for binding to the surface of individual microparticles, and en ugh for measurement from individual micro-particles by means f a labelled sp cific antibody (labelled bioaffinity reactant) and with the sensitive label technology used. Th measurem nt rang required and th sensitivity f the measur ment will be controlled

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by adjusting th amount f microparticles used in the assay and by adjusting sample v lum , if ne ded.

Oth r specific bioaffinity assays such as nucl ic acid hybridizati n assays, ligand-l ctin assays and lig-and-receptor assays are comparabl to th immunoassay methods described abov .

Individual microparticles can be assayed with .g. a flow cytometer, time-resolved microscope r time-resolved microfluorometer or with other measuring instruments based on the use of time-resolved technology (US 5,028,545; Seveus L et al., Cytometry 13: 329 - 338 (1992).

Microparticles may also eventually be used in multiparameter assays, in which several analytes are simultaneously assayed in the same sample volume. In the multiparameter assay a mixture of microparticles is used, in which the particles assaying for different analytes are coated with analyte-specific bioaffinity reactants. When the measurement is taken the analyte-specific microparticle categories are identified on the basis of e.g. size, fluorescence, time-resolved fluorescence, chemiluminescence or bioluminescence. The multiparameter assays using microparticles, based on a bioaffinity reaction are performed according to the present method. In the case of several analytes being assayed simultaneously the amount of the analyte-specific microparticles used in the assay is adjusted according to analyte following the above principles.

The present invention will also be illustrated by the following non-restrictive examples.

Example 1

Quantitation of label (fluorescent Eu chelate) per microparticle and immunoassay measurement from individual microparticles using as a model the non-competitive hCG assay and measurement based on time-resolved fluorescence.

The hCG (hCG = human chorionic gonadotrophin) assay has been performed as a non-competitive immunoassay with a specific monoclonal antibody against the β subunit being biotinylated and bound to 20 μm microparticles coated with streptavidin. The labelled bioaffinity reactant has been a monoclonal antibody labelled with a fluorescent Eu chelate, specifically recognizing the α subunit of hCG. The immunoassay has been performed in one step, during which the antibody-coated microparticles, the hCG calibration standard (2 - 10,000 U/l, where U/l = unit/l) and the labelled antibody are all present simultaneously. After the immunoassay the number of microparticles has been checked by counting, and the Eu concentration has been determined from the known microparticle amount by using the DELFIA assay solution, which then allows the calculation of the amount of Eu molecules per microparticle. In addition individual microparticles have been measured by time-resolved microfluorometry which allows the measurement of the concentration per microparticle of the hCG-specific antibody labelled with fluorescent Eu chelate. The hCG assay presented as an example has not been completely optimized.

Results: Table 1 shows the results of the hCG assay when using 20 μm microparticles. In these assay conditions the lowest hCG concentration resulted in about 5,000 Eu molecules per microparticle, the variation within the experiment still being wide. In addition the measuring range is at least 1,000-fold. Figure 1 shows a calibration curve for hCG construed by measuring individual microparticles by time-resolved microfluorometry.

Example 2

The effect of the amount of microparticles coated with antibody (bioaffinity reactant) on the sensitivity of the assay using as a model the non-competitive hCG immunoassay and the measurement of individual microparticles with a microfluorometer, based on time-resolved fluorescence.

The immunoassay of hCG was performed according to the preceding example. A total volume of 20 μl, three concentrations of calibration standard (0, 250, and 2,500 U/l) and three different amounts of microparticles were used in the assay. After the immunoreaction individual microparticles were measured by time-resolved microfluorometry.

Results: Table 2 and Figure 2 show the effect of the amount of microparticles on the signal strength (photons/s/particle) measured from individual microparticles; with a smaller amount of microparticles the hCG (analyte) in the sample will be bound in a higher concentration onto the surface of individual microparticles; the sensitivity of the assay and the measurement range may be controlled, if needed, by the amount of the microparticles and the sample volume, so as to allow measurement from individual microparticles. The reproducibility of th assay can b improved by measuring a greater number of individual microparticles.

Example 3

The effect of the amount of microparticles on nucl i acid hybridizati n reaction using two differ-

nt   ncentrations of target nucleic acid.

The microparticles, of a diameter f 20 μm, were coated with streptavidin and used in a hybridizati n assay where th targ t was bi tin-labelled synthetic oligonucl otid and the probe a synthetic lig nucleotid labelled with a fluorescent Eu ch late. The xperiment was perf rmed using two diff r nt amounts of targ t ligonucleotide (10^9 and 10^{10} molecules). The total assay volume was 20 μl and 2.0 ng of probe labelled with the fluorescent Eu chelate was used in each assay. The amount of microparticles used per experiment varied from 10 - 10,000 for each target oligonucleotide concentration. After the hybridization reaction the Eu concentration was measured from the surface of the microparticles by time-resolved microfluorometry.

Results: Table 3 and Figure 3 show the effect of the amount of microparticles on the signal strength measured from individual microparticles; with a smaller amount of microparticles the amount of the target oligonucleotide in the sample will bind in higher concentration onto the surface of individual microparticles; again the sensitivity of the assay and the measurement range may be controlled, if needed, by the amount of microparticles used and by the sample volume so as to allow measurement from individual microparticles.

A specialist in the field will appreciate that the different applications of the said invention may vary within the scope of the claims. It will be appreciated that the methods of the present invention can be incorporated in the form of a variety of embodiments, only a few of which are disclosed herein. It will be apparent to the artisan that other embodiments exist and do not depart from the spirit of the invention. Thus, the described embodiments are illustrative and should not be construed as restrictive.

TABLE 1

Calculated Eu amounts in hCG-standards using 20 μm particles. Great assay amount.

hCG U/l	signal strength photons/s	particles	Eu fmol	Eu fmol/cm²	Eu mol/part.	Eu molecules/particle	average	% CV
2	118	9624	0.082	0.678	8.52×10^{-21}	5130	5130	
	<0	10451						
	<0	7444						
10	130	7970	0.09	0.899	1.13×10^{-20}	6805	13801	72.09
	<0	6917	0.212	2.77	3.48×10^{-20}	20957		
	306	6090						
100	817	7293	0.566	6.176	7.76×10^{-20}	46732	58696	20.21
	869	6165	0.603	7.783	9.78×10^{-20}	58897		
	1030	8195	0.714	6.933	1.17×10^{-19}	70459		
1000	12955	7293	9.98	97.99	1.23×10^{-18}	740727	618677	17.21
	13142	9624	9.11	75.33	9.47×10^{-19}	570299		
	11474	8797	7.96	72.01	9.05×10^{-19}	545006		
5000	78247	5940	54.26	726.92	9.14×10^{-18}	5504263	4420302	21.13
	71234	7820	49.4	502.7	6.32×10^{-18}	3806011		
	70166	7368	48.66	525.55	6.60×10^{-18}	3974632		
10000	146072	6090	101.29	1323.55	1.66×10^{-17}	9996802	6989043	45.32
	112916	7368	78.3	845.67	1.06×10^{-17}	3683500		
	113853	6541	78.95	960.5	1.21×10^{-17}	7286826		

CV = coefficient of variation

TABLE 2

Effect of th amount of microparticl s on th signal strength			
hCG U/l	particles	signal strength photons/s/part.	CV %
0	100	252	6.5
250	100	490	26.15
2500	100	1671	23.1
0	1000	277	10.39
250	1000	345	15.76
2500	1000	418	19.98
0	10000	278	7.05
250	10000	366	23.34
2500	10000	401	38.5

Total assay volume 20 µl, of which standard = 5 ml. Size of microparticles 20 µm. Microfluorometry, laser excitation, attenuation 1:100.

TABLE 3

amount of particles	amount of analyte molecules	signal strength photons/s/part.	CV %
10000	10^9	497	34
1000	10^9	1005	48
100	10^9	1885	22
10	10^9	3852	24
no analyte/100		1358	40
10000	10^{10}	1622	31
1000	10^{10}	5613	25
100	10^{10}	13236	33
10	10^{10}	15594	45
no analyte/100		1887	43

Claims

1. A biospecific assay method in which
   - micropartid s coated with th bioaffinity reactant A binding the analyte to be assay d; the sampl
     to be analyzed, and the labell d bioaffinity reactant B are mixed, thus initiating th binding reacti n
     b tween th bioaffinity reactant A, bi affinity reactant B and th analyte,

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- the signal strength from the labell d bioaffinity reactant B bound to th microparticles is quantitated for th d termination of th analyte concentration in th sampl , **characterized**
- by th use in th assay of such an amount of sampl and microparticles that aft r binding of th analyte of the sample t th said amount of microparticles, each individual microparticle will emit such a signal strength as to allow the measurement of the analyte concentration of the sample over the whole range of typical analyte concentrations, and
- by separate measurement of the signal strength from each microparticle.

2. The assay method according to claim 1, **characterized** by the adjustment of the amount of microparticles so as to allow the measurement of the lowest analyte concentration in the sample from individual microparticles by the sensitive label technology used.

3. The assay method according to claim 1, **characterized** by the adjustment of the amount of microparticles so as to allow the measurement of the highest analyte concentration in the sample from individual microparticles by the sensitive label technology used.

4. The assay method according to claim 2, **characterized** by the use of an increasing sample volume in the non-competitive assay and in the competitive assay.

5. The assay method according to claim 3, **characterized** by the use of a decreasing sample volume in the non-competitive assay and in the competitive assay.

6. The assay method according to claim 1, **characterized** by the assay being a non-competitive immunoassay, in which the labelled bioaffinity reactant B is an antibody directed against the antigen of the analyte.

7. The assay method according to claim 1, **characterized** by the assay being a nucleic acid hybridization assay, in which the labelled bioaffinity reactant B is a nucleic acid probe.

8. The assay method according to claim 1, **characterized** by the assay being a competitive immunoassay, in which the labelled bioaffinity reactant B is an antigen, and the bioaffinity reactant A an antibody, for whose binding sites the labelled antigen and the antigen of the analyte compete.

9. The assay method according to claim 8, **characterized** by the control of the amount of microparticles coated with the antibody A so that the lowest analyte concentration will result in the strongest signal, when measuring individual microparticles by the label technology used.

10. The assay method according to claim 1, **characterized** by the use of labels emitting fluorescence, time-resolved fluorescence, chemiluminescence or bioluminescence.

11. The assay method according to claim 1, **characterized** by the microparticles used being a mixture of microparticles recognizing different analytes, thus allowing the simultaneous assay of several analytes in the same sample.

12. The assay method according to claim 11, **characterized** by the identification of the microparticles recognizing different analytes by using fluorescence, time-resolved fluorescence, chemiluminescence or bioluminescence or their combinations.
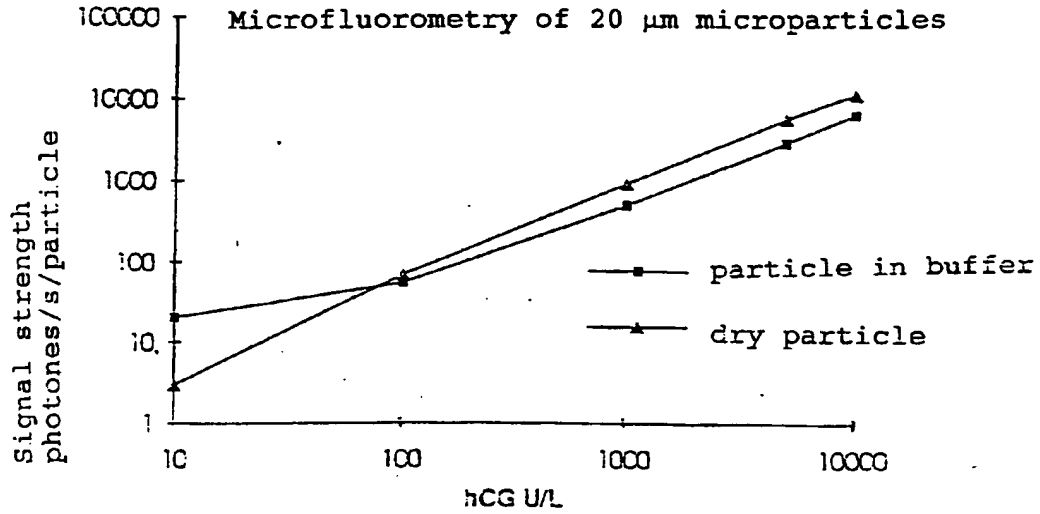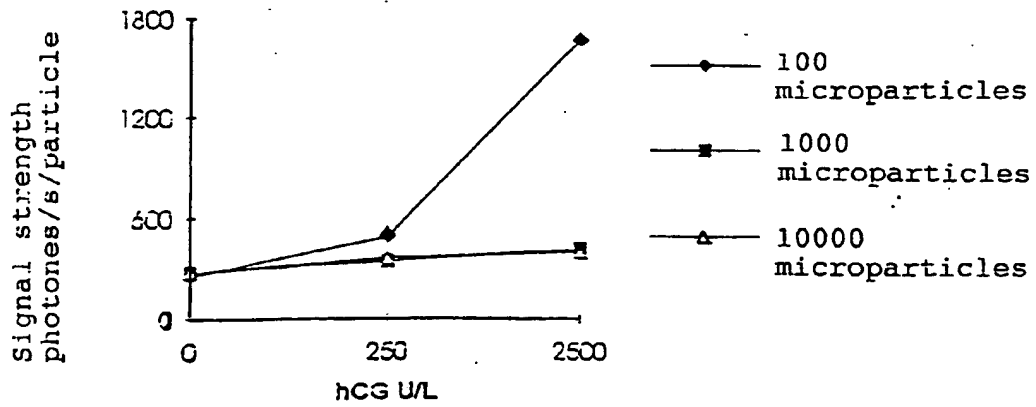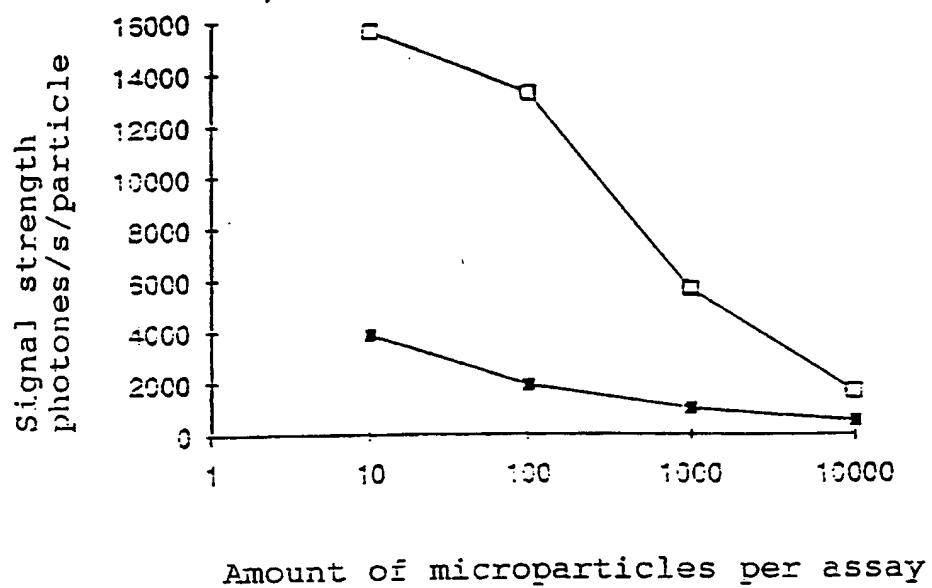
FIG. 1

FIG. 2

FIG. 3